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Human Immunodeficiency Virus Type 1 Two-Long Terminal Repeat Circles: A Subject for Debate

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Abstract

HIV-1 infections are characterized by the integration of the reverse transcribed genomic RNA into the host chromosomes making up the provirus. In addition to the integrated proviral DNA, there are other forms of linear and circular unintegrated viral DNA in HIV-1-infected cells. One of these forms, known as two-long terminal repeat circles, has been extensively studied and characterized both in in vitro infected cells and in cells from patients. Detection of two-long terminal repeat circles has been proposed as a marker of antiretroviral treatment efficacy or ongoing replication in patients with undetectable viral load. But not all authors agree with this use because of the uncertainty about the lifespan of the two-long terminal repeat circles.

We review the major studies estimating the half-life of the two-long terminal repeat circles as well as those proposing its detection as a marker of ongoing replication or therapeutic efficacy. We also review the characteristic of these circular forms and the difficulties in its detection and quantification. The variety of approaches and methods used in the two-long terminal repeat quantification as well as the low reliability of some methods make the comparison between results difficult. We conclude that it is not possible to draw a clear supposition about the lifespan of two-long terminal repeat circles and consequently they should not be used as a marker of ongoing replication without a careful analysis of the methods and results. **(AIDS Rev. 2016;18:23-31)**

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Key words

HIV-1. Virus replication. Viral DNA. Circular DNA. HIV-1 LTR.

Introduction

The most characteristic step of the replicative cycle of retrovirus is the conversion of the viral genomic RNA to cDNA (a linear double-stranded DNA) carried out by the viral reverse transcriptase. The cDNA is transported into the nucleus in a pre-integration complex and integrated into the host cell genome by the action of the viral integrase[1]. This integrated viral cDNA is designated proviral DNA. In addition to the proviral DNA, cells infected by retrovirus contain several forms of unintegrated viral DNA, mainly linear DNA, two-long terminal repeat (2-LTR) circles, and 1-LTR circles (Fig. 1).

The existence of unintegrated DNAs in cells infected by retroviruses was first described in cells infected by avian sarcoma virus[2] or Moloney murine leukemia virus[3]. The first characterization of circular unintegrated DNA with 1-LTR or 2-LTR was carried out in the nuclear DNA from cells infected by mouse mammary tumor virus and avian sarcoma virus[4,5].

Unintegrated HIV-1 DNA was first identified in CEM and H9 infected cells or in peripheral blood mononuclear cells (PBMC) infected *in vitro*[6,7]. The characterization of the 1-LTR and 2-LTR forms was performed in CEM and U937 cells infected by HIV-1[7,8]. In HIV-1-infected patients, the presence of unintegrated DNA was

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detected in activated PBMCs[9], in resting CD4+ T-cells from lymph nodes or blood[10], and in other cells such as tissue macrophages in the brain[11].

Although the 2-LTR circles are the less abundant form of unintegrated viral DNA in infected cells[8], they have become the most widely studied due to the difficulty of detection and quantification of the other unintegrated DNA forms.

In HIV-1 patients, much effort has been made in order to use the presence of 2-LTR circles as a prognostic marker of the infection[12,13]. The existence of 2-LTR circles in HIV-1 patients on long-term antiretroviral therapy is considered an evidence of recent viral replication[12,14]. This statement assumes that 2-LTR circles have a short lifespan, and thus their presence can only be caused by recent viral replication[15,16]. However, there is a great polemic in relation to the lifespan of 2-LTR circles because other authors think that 2-LTR circles can persist for a long time in cells[17,18]. The estimation of the 2-LTR circles half-life has been performed by different methods, making the comparison between studies difficult. Quantification of 2-LTR circles is especially problematic when the template concentration is low, as in the case of HIV-1-treated patients[19]. The aim of the review is to discuss the controversy about the lifespan of 2-LTR circles and of its use as a marker of ongoing replication in patients.

Mechanisms of generation of circular viral DNA

All circular HIV DNAs are derived from the linear DNA that is made circular by different mechanisms[20]. The 2-LTR circles are formed by the joining, in the nucleus, of both ends of the linear DNA by cell ligases[20-22]. The formation of the 2-LTR circles seems to be related to the protection of the cells for the toxicity of the double-stranded ends of the unintegrated linear DNA. Indeed, the host cell non-homologous DNA end joining (NHEJ) pathway, which repairs double strand breaks, is involved in the 2-LTR circle formation[23]. Specifically, the NHEJ factors Ku protein, ligase 4, and XRCC4 have been implicated in this mechanism[23].

It is generally assumed that 1-LTR circles are formed by recombination of the LTRs from the linear DNA, and a model for its formation based in *in vitro* experiments was proposed[20]. The 1-LTR circles could also arise from incomplete products from reverse transcription[24]. In fact, a significant proportion (about 10%) of the 1-LTR circles are formed in the cytoplasm, coincident with reverse transcription[22]. At present, why 1-LTR circles are formed is unknown, but the Rad50/Mre11/NBSI complex, also implicated in DNA repair, was involved in its formation[25].

In cells infected by retroviruses, in addition to 2-LTR and 1-LTR circles, other circular forms with different size or with LTRs in an incorrect position are found. These irregular circles seem to be formed by autointegration[8,21], that produces a diversity of forms like nicked inverted and subgenomic DNA circles (Fig. 1)[21].

Long terminal repeat circle junctions

The boundary between the two LTRs in 2-LTR circles is named circle junction. The linear DNA product of reverse transcription has a dinucleotide added at each LTR end (GT at the 3' and AC at the 5') relative to the RNA sequence (Fig. 1). For integration, the viral integrase deletes a GT dinucleotide at each 3' end of the linear DNA (endo-nucleotide cleavage known as 3' processing)[26]. In HIV-1, the blunt end ligation of both ends of linear DNA produce the junction sequence CAGTACTG, where GT and AC are the dinucleotides added (Fig. 1). This sequence, named canonical junction sequence, is the most frequently found in 2-LTR circles. Circle junction sequences lacking some of these nucleotides are also found because of the circularization of total or partially processed linear DNA forms. Moreover, there are a variety of other junction sequences with insertions or deletions of diverse sizes or sequences coming from defective linear molecules unable to integrate[27-30] (Fig. 2). Insertions often contain a part of the primer binding site or the polypurine tract (ppt) sequence. This is understood to be caused by the RNase H failure in removing the tRNA primer from the minus strand DNA, or the ppt primer from the plus strand DNA during reverse transcription[28,31]. Indeed, mutations in the viral RNase H modifying the RNase activity increased the number of 2-LTR circle junctions with these insertions[32]. Junction sequences with bigger insertions containing fragments of other regions of the viral genome are also found because of autointegration[21] and they could be considered as a class of molecules distinct from the 2-LTR circles. Deletions in the junction sequence were explained by the degradation of viral DNA ends by host nucleases at one or both ends of the viral DNA before ligation[29]. The amount of canonical sequences is around 50-60% in *in vitro*-infected cells[28,32], but in PBMCs from patients, the fraction of junction sequences with anomalous sequences is higher (66.0-87.5%)[31,33]. Figure 2 shows some examples of the several types of 2-LTR circle junction sequences, and the different mechanisms suggested for their formation.

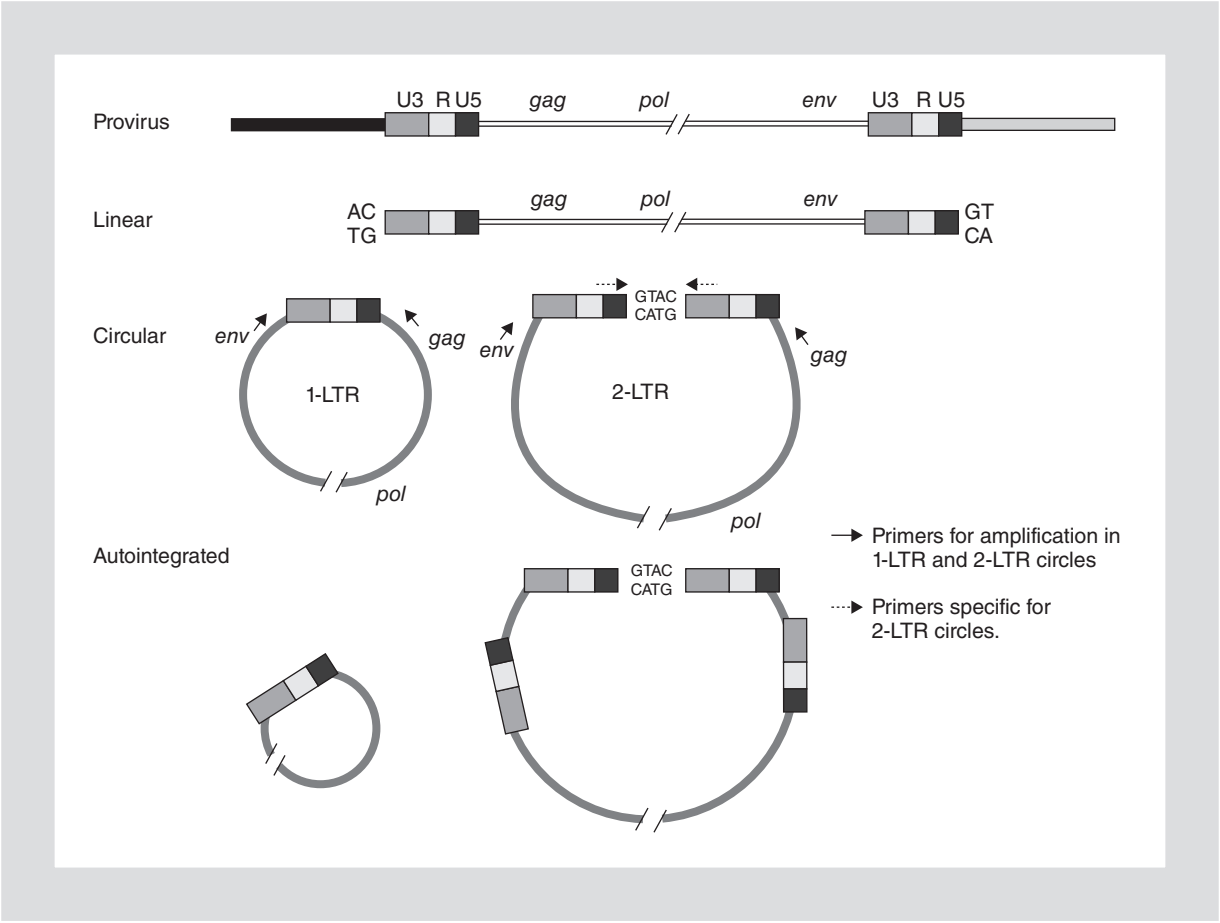

Figure 1. *Viral DNA forms in HIV-1-infected cells. The sequence of the dinucleotide added at each long terminal repeat end, which constitute the canonical junction sequence in 2-LTR circles, appears in linear DNA and 2-LTR circles. Position of the primers used for amplification of circular DNA forms is shown.*
LTR: long terminal repeat.

Methods for the detection and quantification of two-long terminal repeat circles

The first descriptions of unintegrated retroviral DNA as well as the characterization of 1-LTR and 2-LTR circles were made by Southern blot analysis with DNA from retrovirus-infected cells. Fragments corresponding to 1-LTR and 2-LTR circles were identified by their migration in agarose gels and characterized by restriction endonucleases digestion[4,6,7].

Since 1990, all studies aimed at the detection and quantification of unintegrated circular DNA, were performed by a specific polymerase chain reaction (PCR), using primers whose orientation permit only the amplification of the circular DNA[34,35]. Sometimes, PCR primers hybridizing in *gag* and *env* genes were used for the detection of both 1-LTR and 2-LTR circles[15] (Fig. 1). But these primers often produce false positive amplifications since they can amplify other DNA forms, as for example linear unintegrated or proviral DNA[36]. A specific PCR for the detection of 2-LTR circles with primers hybridizing inside the LTR is more reliable. In theory, primers that span the 2-LTR circle junction can only amplify 2-LTR circles; however, the products obtained after amplification with these primers sometimes do not correspond to 2-LTR circles but to other DNA circles formed by autointegration[20] or to artifacts made during amplification[37]. Limited amounts of target DNA contribute to the generation of unspecific products during PCR[38].

Quantification of 2-LTR circles has been performed by several techniques (Table 1); (i) specific PCR followed by Southern blot using an internal radioactive probe[18,34,37]; (ii) real-time quantitative PCR using fluorescent cyanine dyes[35,38,39]; (iii) real-time quantitative PCR using TaqMan probes[40,41]; and (iv) droplet digital PCR (ddPCR)[19,42].

Real-time PCR techniques are the most widely used for 2-LTR circles quantification. They require for quantification of PCR products the development of a standard curve with known amounts of the amplicon per reaction.

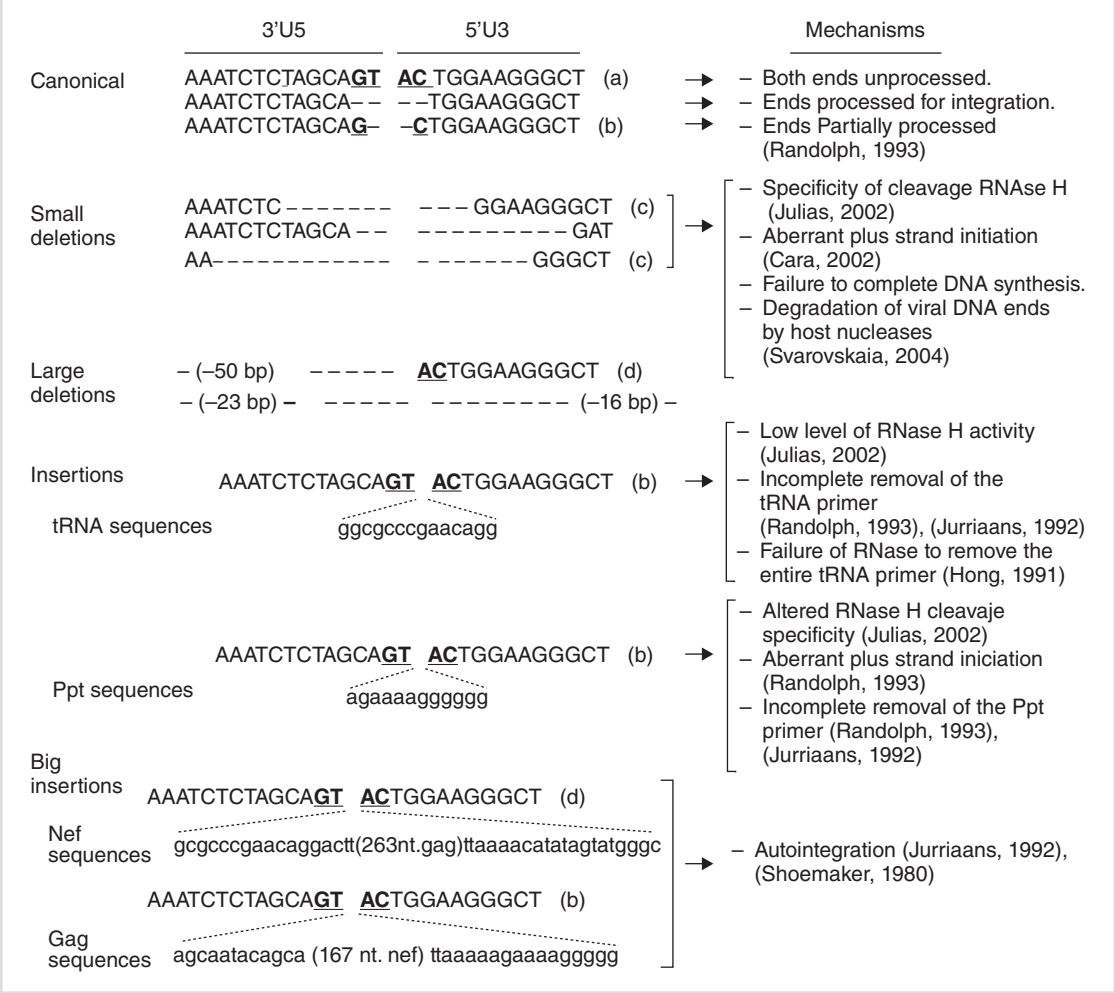

Figure 2. *Types of two-long terminal repeat circles junction sequences. One example of each type of known junction sequences is shown. Sequences were obtained from:* ***(a)*** Hong, et al.[28]*;* ***(b)*** Jurriaans, et al.[31]*;* ***(c)*** Olivares, et al.[71]*; and* ***(d)*** Svarovskaia, et al.[29]. **Bold letters mark the canonical dinucleotide junction sequence. Mechanisms proposed by the formation of each type are listed. Ppt: polypurine tract.*

For this purpose, serial dilutions of a plasmid containing a DNA fragment with the canonical junction sequence of 2-LTR circles are usually employed. Optimization of the techniques using plasmid dilutions provides a good efficiency and reliability. However, amplification of 2-LTR circles from *in vitro* infected cells and from infected patients' PBMCs is affected by viral sequence variability, circle junction variability, the presence of different DNA forms in the same sample, and the low proportion of 2-LTR circles relative to total cellular DNA. Although conserved sequences are selected in the design of primers and probes for the 2-LTR circle-specific PCR, mutations and deletions in the 2-LTR circles can cause a poor annealing and an underestimation of the 2-LTR circles. On the contrary, artifacts produced from the amplification of other DNA forms could lead to false positives and higher values. Indeed, in some quantitative PCR reactions, products were analyzed on agarose gels or submitted to sequence analysis to ensure their specificity[18,40]. All these problems are more evident when low amounts of 2-LTR circles exist, as in the case of treated patients. In studies comparing quantification of 2-LTR circles by real-time PCR and by ddPCR, a low reliability was obtained with values < 300 copies/million cells using real-time PCR. Droplet digital PCR resulted in a 20-fold increase in accuracy, suggesting that this technique could prove the most useful for 2-LTR circles quantification in cells from patients[19]. Moreover ddPCR

Table 1. Methods for the detection and quantification of two-long terminal repeat circles

Method	Detection	DNA	Specificity	References
Real-time PCR	TaqMan probe	Episomal*	2-LTR	Sharkey, et al.[40]; Brussel, et al.[43]
Real-time PCR	TaqMan probe	Total cellular	2-LTR	Butler, et al.[17]; Gillim Ross, et al.[47]
Conventional single PCR	Southern blot P^{32} labeled probe	Total cellular	2-LTR	Pierson, et al.[18]
Real-time PCR	SYBR Gold	Episomal†	2-LTR	Teo, et al.[35]
Real-time PCR	TaqMan probe	Total nuclear	1-LTR and 2-LTR	Kelly, et al.[44]
Conventional single PCR	Southern blot Phosphatase alkaline labeled probe	Total cellular	2-LTR	Clarke, et al.[51]
ddPCR	TaqMan probe	Total cellular	2-LTR	Hatano, et al.[14]
Real-time PCR	SYBR green	Episomal‡	2-LTR	Casibianca, et al.[75]
Pre-amplification and real-time nested PCR	TaqMan probe	Cell lysates	2-LTR	Vandergeeten, et al.[76]
ddPCR	TaqMan probe	Episomal*	2-LTR	Malatinkova, et al.[46]

*Episomal DNA was obtained from cell pellet using QIAprep Miniprep Kit (Qiagen).
†Cell lysates were first digested with proteinase K. Episomal DNA was obtained using QIAprep Miniprep Kit and the remaining genomic DNA was removed by Plasmid-Safe DNase digestion.
‡Episomal DNA obtained from 5 µg of total cell DNA using QIAprep Miniprep Kit.
PCR: polymerase chain reaction; ddPCR: droplet digital PCR; LTR: long terminal repeat.

provides an absolute quantification of target sequences without relying on the use of standard curves[19].

In addition to the PCR, another factor affecting the 2-LTR circles quantitation is the origin of the DNA used in the amplification. One of the most important problems for the 2-LTR circles amplification is the low rate of these forms among a large background of host cellular DNA. This low quantity requires the use in the PCR of a great amount of DNA, which can inhibit the PCR reaction[19]. Methods for the elimination of high molecular DNA, as the Hirt DNA extraction method, were used in 2-LTR characterization studies in order to increase the episomal concentration[6-8,21,27]. Also, plasmid DNA isolation methods that remove bacterial genomic DNA were employed for the separation of episomal DNA in *in vitro* and *ex vivo* studies[35,39,40,43], whereas in other works, high molecular DNA was not removed[18,19,44].

Although the recovery of 2-LTR circles DNA seems clearly enriched using plasmid isolation methods[40], there are conflicting results regarding the efficacy of these methods to obtain 2-LTR circles. In a study comparing the impact of different extraction methods on the recovery of 2-LTR circles, bacterial plasmid extraction recovered only 10-20% of the 2-LTR circles DNA, whereas total DNA extraction achieved about 45% of the 2-LTR circles[45]. However, others authors found higher levels of recovery (1.9-fold) with plasmid extraction methods than with total DNA extraction[46]. A drawback of the bacterial plasmid extraction method is the wide variation observed in the isolation efficiency between experiments, making a normalization necessary[46]. Table 1 summarizes some of the differential characteristic of the methods used for the detection of 2-LTR circles.

Lifespan of two-long terminal repeat circles

A great controversy about the lifespan of 2-LTR circles exists. On the one hand, 2-LTR circles have been proposed to have a short lifespan because they are degraded by cellular enzymes. On the other hand, 2-LTR circles could be as long lasting as other circular DNA forms. Several *in vitro* and *in vivo* studies, using different approaches and methods, have been performed for the resolution of this issue, but with discordant conclusions. The first studies were made in cell culture, measuring the decay of 2-LTR circles; in HIV-1-infected PBMC or

CEM cells a half-life of 16 days was estimated[37]. In another work, in favor of the lability of 2-LTR circles, MT-4 or Jurkat cells were infected with an HIV-1 isolate and after 24 hours a reverse transcriptase inhibitor was added to avoid further rounds of virus infection. In this experiment, a rapid decrease of 2-LTR circles in both cell types was observed[40].

Two main papers have been published against the lability of 2-LTR circles[17,18]. In the first one, SUP-T1 cells were infected with HIV-1, adding a protease inhibitor to restrict viral replication to a single cycle; the decrease in 2-LTR circles correlated with the death of the infected cells and with the culture dilution[17]. In the second work, MT-2 cells were infected and after 48 hours cells were treated with indinavir; the decay of 2-LTR circles, cell death, and cell division were carefully measured, and the decay of the 2-LTR circles was very similar to the inverse of the doubling time of the cells, indicating that the dilution factor is responsible for the decay[18]. In order to exclude the dilution factor, *in vitro* experiments infecting monocyte-derived macrophages were performed because these cells do not divide. In Teo, et al.[35], the half-life for 2-LTR circles was 19.2 hours, while in two similar studies using an integrase-defective virus, they did not find a decay of the 2-LTR circles after 21 days[47] or 30 days post-infection[44].

Recently, the stability of 2-LTR circles was examined in primary naive CD4+ T-cells infected *in vitro* and cultured for a month. Since T-cell receptor excision circles (TREC) are accepted to be stable, the stability of 2-LTR circles was compared with the stability of TRECs and with the integrated HIV DNA. In conditions where naive cells do not divide nor decrease viability, 2-LTR circles and TRECs were stable for 30 days of culture, suggesting that both forms are stable[48].

In vivo studies to investigate the lifespan of 2-LTR circles have often been made in HIV-1-infected patients after antiretroviral treatment. In PBMCs from four HIV-1-infected patients, after adjustments for antiretroviral treatment, Sharkey, et al.[40] found that decreases in plasma viral load were accompanied by a considerable decline in 2-LTR circle copy numbers, in contrast with a lower diminution of total viral DNA levels; the authors concluded that 2-LTR circles are labile *in vivo*. This result was supported by another study where in seven out of nine patients, after the beginning of antiretroviral treatment, viral load decrease correlated with a decrease of 2-LTR circles[39]. However, with a similar approach in 11 HIV-1 patients in whom antiviral treatment led to a rapid decrease in viral load, no decrease of 2-LTR circles was found[43].

With a different approach, the lability of 2-LTR circles was evaluated in 11 patients after the initiation of treatment by comparing the emergence of the drug resistance mutation to lamivudine (M184V) in plasma RNA, episomal DNA, and proviral DNA. A complete replacement of the wild-type sequence with the M184V mutation was observed in the plasma viral RNA and in the episomal DNA, while proviral DNA remained wild-type. This result suggested that the wild-type 2-LTR circles were degraded[49].

More recent studies calculated the half-life of 2-LTR circles *in vivo* by monitoring for 100 days after the beginning of treatment 2-LTR circles, integrated DNA, and total DNA levels[41]. The study was performed in two groups of HIV-1 patients with primary and chronic HIV-1 infection. Integrated and total DNA levels were greater in the chronic infection because they accumulated over time, while no differences in 2-LTR circle levels were observed between primary and chronic HIV-1 infection, suggesting that 2-LTR circles do not accumulate because of their lability. Moreover, consistent with the use of an integrase inhibitor, a rapid increase of 2-LTR circles was detected at the initiation of therapy; this increase was followed by a decay, with a half-life of about 25 days that supports the lability of this form. However, a slower second-phase decay was observed with a half-life of 169 days[41]. The same authors in a different study, analyzing several subsets of CD4+ T-cells after the start of therapy, estimated a half-life of the 2-LTR circles at around 12-52 weeks[50].

In an interesting work studying the replication dynamic of the simian immunodeficiency virus (SIVmac) in macaques, it was possible to analyze several lymphoid tissues (spleen, mesenteric lymph nodes, peripheral lymph nodes, and thymus). In the peripheral blood cells, 2-LTR circles levels increased up to 28 days post-infection, but they disappeared at 42 days post-infection. However, at this time, high levels of 2-LTR circles were detected in lymphoid tissues. The difference between the levels of 2-LTR circles in different body compartments cautions against conclusions from *in vivo* experiments[51].

The different approaches and methods used in all reviewed works make difficult the comparison between studies and could influence the different conclusions. Based in the analysis of all these studies, we cannot draw a clear conclusion about the lifespan of the 2-LTR circles.

Practical use of two-long terminal repeat circles detection in HIV-1-infected patients

Two-LTR circle quantification was proposed as a marker of antiretroviral drug therapeutic efficacy[13]. In *in vitro* studies, after treatment of infected cells with antiretroviral

drugs acting on different steps of viral replication, a decrease of 2-LTR circles was observed[52]. *In vivo*, while total and integrated DNA levels were stable in a high proportion of patients during early stages of HAART, a rapid decrease of 2-LTR circles level was observed[53]. Puertas, et al.[54] supported the efficacy of maraviroc added to triple therapy because of a decline of 2-LTR circles.

In vitro, the integration block with integration-defective virus[55] or by treatment with integrase inhibitors lead to an increase in the 2-LTR circle levels[29,56]. This result is probably related to the fact that if integration is inhibited, there will be more DNA available for circularization. In patients treated with raltegravir, an increase of 2-LTR circle levels was observed soon after treatment; this increase was transient and followed by a decrease as a result of a decrease in viral replication[12,14].

Some authors have questioned the use of 2-LTR circles as a marker of therapeutic efficacy[43,57-59]. Studies with patients after months on HAART or submitted to structured interruption of antiretroviral therapy found a low impact in 2-LTR circle levels[43,57]. Also, in patients treated with integrase inhibitors, no change in 2-LTR circle levels was observed[58,59]. Diverse criteria for the selection of HIV-1 patients as well as the type of treatment, previous treatments, or viremia can be responsible for the distinct results. Also, the different time of sample recovery and the techniques used for the quantification of 2-LTR circles can affect the results of the study.

A possible prognostic value of 2-LTR circle levels in HIV-1-infected patients was investigated since they were related to clinical progression, CD4+ T-cell levels, and plasma viremia[9,37]. However, no clear conclusion was reached[60]. Sequence analysis of episomal DNA was proposed for the prediction of the virus that rebound after therapy interruption since the viral rebound RNA sequence was phylogenetically related to the episomal but not to the proviral sequences present before treatment interruption[61].

Long terminal repeat circles as a marker of ongoing replication

The origin and the mechanisms responsible for the residual viremia in HIV-1-infected patients after prolonged HAART still remain unclear[62]. Since HIV-1 persists as a latent provirus in resting memory CD4+ T lymphocytes[63], a first possibility was that residual viremia is the consequence of the reactivation of these cells. Some authors have questioned this origin[64,65] and they propose a low-level continuous viral replication in an unknown reservoir. Presence of 2-LTR circles in HIV-1 patients on long-term treatment has been often used to support the existence of ongoing replication[15,16]. More strong evidence of ongoing replication has been suggested by the transient increase in 2-LTR circle levels after drug intensification with raltegravir in patients previously treated with HAART[12,14]. For the explanation of the formation of 2-LTR circles in the presence and absence of raltegravir intensification, a mathematic model was developed[66]; this model was validated against patient data from the Buzon study[12]. According to this model, a transient increase in 2-LTR circle concentrations after raltegravir intensification is consistent with significant levels of efficient de *novo* infection cycles[66].

The evidence of ongoing replication based in 2-LTR circle levels was criticized by others[67]. They claimed that the changes in 2-LTR circle levels after raltegravir intensification were not followed by changes in viral load, as would occur if treatment with raltegravir inhibited residual viral replication. Also, in a group of patients monitored for 10 years after HAART, a similar decay pattern of 2-LTR circles and total HIV-1 DNA was detected; this, along with the persistence of 2-LTR circles throughout the follow-up, suggests that 2-LTR circles may persist in long-lived cells and are not necessarily an indicator of ongoing HIV-1 replication[68,69].

Research applications

In spite of the conflict about the use of 2-LTR circles as a diagnostic or prognostic marker, its quantification and the analysis from junction sequence has been useful in many HIV-1 research studies. For example, mutations in the polypurine tract affecting HIV-1 replication[70] or mutations in the RNase H affecting its specificity[32] were characterized by analysis of the 2-LTR circles junction sequence. Also, in the exploration of the mechanism of action of the integrase inhibitors azido-containing β-diketo acid derivatives, analysis of the 2-LTR circle junction sequence was used[29]. Ongoing replication in a persistently HIV-1-infected cell line was evidenced by the existence of 2-LTR circles[71].

In studies exploring the mechanisms responsible for the control of viremia in elite controllers, measurements of 2-LTR circles were also performed. These patients were found to have lower levels of integrated DNA and higher levels of 2-LTR circles compared to patients on HAART[72]. Due to this result, it was proposed that in elite controllers there was an integration inhibition. However, attempts to demonstrate this inhibition in *ex vivo* infection of PBMCs from elite controllers resulted in conflicting results[72,73].

Conclusions

Much effort has been made to understand the significance of unintegrated DNA forms in HIV-1-infected cells. Despite that 2-LTR forms are the less abundant of all unintegrated viral DNA, they have been the most widely studied[8,63,74]. This is because the characteristic junction sequence makes possible the use of a specific PCR for their detection, while the study of other unintegrated DNA forms by PCR produces false positives[36].

Regarding the quantification of 2-LTR circles, the difficulty comes from the low proportion of these forms among the host cellular DNA background. In addition, viral sequence and circle junction variability with the presence of insertions and deletions makes a significant proportion of sequences undetectable. In spite of this, in *in vitro* infected cells there is enough unintegrated DNA to perform the detection and quantification, and the study of the junction sequences in 2-LTR circles has been fruitful in many HIV-1 research studies[29,32,70,71].

In HIV-1-infected patients, there have been many attempts to relate the level of 2-LTR circles with different aspects of infection, such as the estimation of treatment efficacy, the detection of ongoing replication in treated patients, or as a prognostic marker of clinical evolution. But quantification of 2-LTR circles in samples of HIV-1-infected patients is more problematic than in *in vitro*-infected cultures since levels of 2-LTR circles in patient samples are often below 50 copies per million PBMCs[12,14,53,72].

Most studies in which conclusions were drawn about the lifespan of 2-LTR circles and the existence or not of viral replication in treated patients were usually performed using real-time PCR for the quantification of 2-LTR circles. This technique does not produce reliable results at template concentrations below 300 copies per million cells, values that are frequent in treated patients[19]. Probably, the use of ddPCR could result in more reliable data, but so far this technique has been rarely used in these studies. Moreover, in a high proportion of studies, episomal DNA was extracted using the bacterial isolation plasmid methods before the diagnostic by PCR. These isolation methods showed a high variation in the efficiency of the recovery of 2-LTR circles and highlight the need for normalization[46]. But these extraction methods have been employed in many studies without an estimation of the recovery efficiency.

The description of all the problems associated with the methods for quantification of 2-LTR circles and the difficult comparison between studies (different methods, contradictory results, lifespan controversy, etc.) caution against the use of 2-LTRs as a marker of ongoing replication in treated patients without a careful analysis of the methods and results.

Declaration of interest

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